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its binding protein might activate different channel types, depending on cell type, extracellular stimulus and perhaps other conditions (Guse, 2012).

However, several studies support a trigger function of NAADP for global  $\text{Ca}^{2+}$  signalling. In both pancreatic acinar and islet cells NAADP rapidly increased after stimulation and preceded an increase in cADPR concentration (Yamasaki et al., 2005; Kim et al., 2008). Jurkat T-lymphocytes stimulated via TCR/CD3 show very similar results since NAADP rapidly increased within seconds and likely initiated local  $\text{Ca}^{2+}$  signals in the so-called pacemaker phase of  $\text{Ca}^{2+}$  signalling (Gasser et al., 2006; Kunerth et al., 2004). Infusion or microinjection experiments in Jurkat T cells showed NAADP evoked  $\text{Ca}^{2+}$  signals in restricted trigger zones (Dammermann and Guse, 2005).

While scanning their environment, the cytoskeleton of T cells undergoes permanent changes allowing for both integrin-dependent and -independent types of motion [reviewed in Krummel and Cahalan, 2010]. While integrin-independent motility is more rapid and dependent on myosin, velocity in the integrin-dependent mode is reduced and thus more cell–cell contacts are possible. The situation changes as soon as antigenic peptide in MHCII context is recognized. Then, T cells may either intensify scanning the APC while motility is reduced, but not fully abrogated. Another possibility is formation of the immune synapse (IS) with rounding of T cells and full stop of motility (Donnadieu et al., 1994). The latter is accompanied by ongoing polymerization and inward streaming of actin into the IS (Kaizuka et al., 2007).

Obviously, velocity of T cell motility depends on integrin interactions, but it is less clear what mechanisms underlie the step from intense scanning at low velocity to T cell rounding and full IS formation.  $\text{Ca}^{2+}$  signalling has been implicated in this process and it has been shown that several proteins involved, including proteins involved in  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  entry, such as Orai1 and Stim1, or the potassium channels  $\text{K}_{\text{Ca}}3.1$ , and  $\text{K}_{\text{V}}1.3$ , co-localize at the IS [reviewed in (Krummel and Cahalan, 2010)].

In this study we used primary rat MBP-specific T cells and the rat astrocyte cell line F10 (in the following termed “astrocytes”) as APC to analyse the role of NAADP during IS formation. Since we previously demonstrated rapid formation of NAADP, we hypothesized that NAADP mediated local  $\text{Ca}^{2+}$  release might regulate early processes of IS formation, e.g., the step from slowly scanning to immotile rounded T cells. Thus, we recorded in parallel changes in  $[\text{Ca}^{2+}]_i$ , cellular shape, and motility of the T cells following contact to astrocytes. The role of NAADP was assessed by blocking NAADP action using the recently validated small-molecule NAADP antagonist BZ194 (Dammermann et al., 2009; Cordiglieri et al., 2010).

## EXPERIMENTAL DETAILS

### Materials

Fura-2/AM was purchased from Calbiochem. DMSO and probenecid were supplied by Sigma. Fibronectin was obtained from Invitrogen. BZ194 was synthesized as described (Dammermann et al., 2009).

### Antigens

Antigen specific T cell clones were specific for guinea pig myelin basic protein (MBP). MBP was purified from guinea pig brains as reported (Eylar et al., 1979).

### Generation and Culturing of T Cells

Rat antigen-specific T cell clones were obtained from lymph node preparations of Lewis rats immunized with MBP. Stimulation, expansion and culture of specific rat T cells were conducted under conditions as described (Flügel et al., 1999).

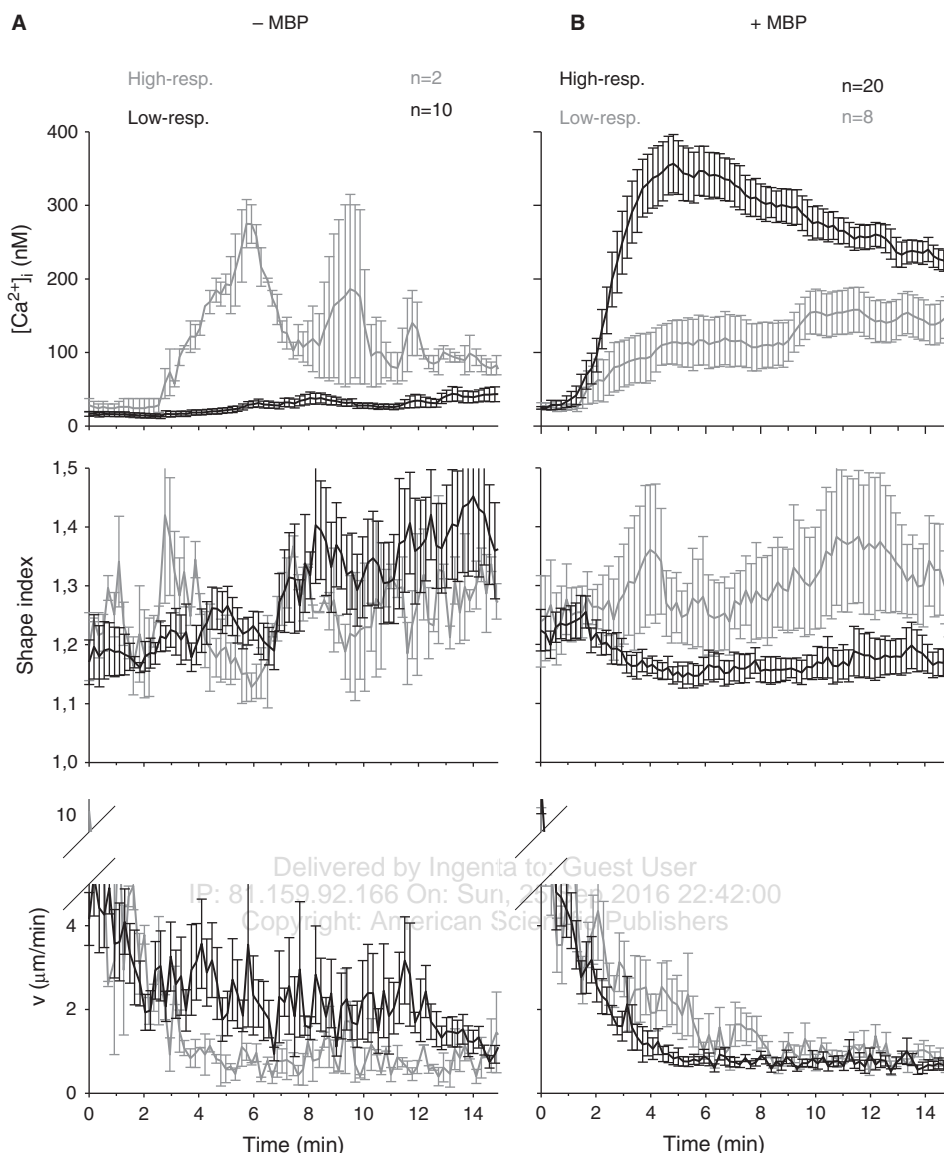
### Analysis of $[\text{Ca}^{2+}]_i$ , Shape and Motility

Rat  $\text{T}_{\text{MBP}}$  cells were loaded with Fura-2/AM as described (Guse et al., 1993) and kept in the dark at  $\sim 15^\circ\text{C}$  until use. Rat F10 astrocytes with up-regulated MHC II (after 48 h-incubation with T cell-blast-conditioned medium) were cultured on  $\mu$ -slides 8 well (ibidi, Martinsried, Germany) on fibronectin and pulsed or not with MBP (10  $\mu\text{g}/\text{ml}$ , 2 hours). Ratiometric  $\text{Ca}^{2+}$  imaging was performed as described recently (Berg et al., 2000). We used an Improvision imaging system (Tübingen, Germany) built around the Leica microscope at 40-fold magnification. Illumination at 340 and 380 nm was carried out using a monochromator system (Polychromator IV, TILL Photonics, Gräfelfing, Germany). Images were taken with a gray-scale CCD camera (type C4742-95-12ER; Hamamatsu, Enfield, United Kingdom) operated in 8-bit mode. The spatial resolution was  $510 \times 672$  pixels. The acquisition rate was  $\sim 1$  ratio in 10 seconds. Raw data images were stored on a hard disk. The images were used to construct ratio images (340/380). Finally, ratio values were converted to  $\text{Ca}^{2+}$  concentrations by external calibration. To reduce noise, ratio images were subjected to median filter ( $3 \times 3$ ) as described previously.<sup>42</sup> Data processing was performed using Openlab software (Improvision, Tübingen, Germany). Image J software (freeware, provided by Wayne Rasband, NIH) was used to evaluate cell shape and velocity. Shape index is defined as  $\text{P}^2/4\pi\text{S}$  (P: perimeter; S: surface of the cell; Donnadieu et al., 1994).

## RESULTS

Fura2-loaded  $\text{T}_{\text{MBP}}$  cells were added to a monolayer of astrocytes and fluorescence images were used to monitor



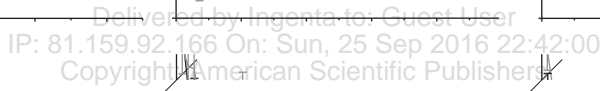


**Figure 2.**  $\text{Ca}^{2+}$  signalling, shape and motility pattern of high- and low-responding  $T_{\text{MBP}}$  cells. Mean  $[\text{Ca}^{2+}]_i$ , shape index and motility of  $T_{\text{MBP}}$  cells after contact to MBP-pulsed (right) astrocytes are displayed ( $\pm$ SEM). Control T cells with non-pulsed astrocytes are shown on the left. Black lines correspond to the majority and grey lines to the minority of T cells at a certain condition (+/- MBP).

$[\text{Ca}^{2+}]_i < 300$  nM in the first ten minutes. This majority of cells, the typical low-responder  $T_{\text{MBP}}$  cells, were low in  $[\text{Ca}^{2+}]_i$  (Fig. 2(A), upper panel), displayed an increased shape index over time (Fig. 2(A), middle panel), and showed motilities between 2 and 3  $\mu\text{m}/\text{min}$  on average (Fig. 2(A), lower panel). Upon presentation of MBP by the astrocytes, the typical high-responder  $T_{\text{MBP}}$  cells were in the majority (Fig. 2(B), upper panel). Further, cellular rounding as judged by a drop in cell shape upon IS formation was observed (Fig. 2(B), middle panel). The  $T_{\text{MBP}}$  cells were virtually “caught” by the astrocytes since also the motility decreased in parallel to cellular rounding (Fig. 2(B), lower panel). Although, low-responders in the presence of antigen started to immobilize, too, this arrest was not as strong as for the high-responders.

Having this cellular assay with three read-outs at hand, the role of NAADP in formation of the IS was analysed. Recently, we showed that the small molecular NAADP antagonist BZ194 blocks local and global  $\text{Ca}^{2+}$  signalling evoked by NAADP, but not by cADPR,  $\text{IP}_3$  or by activation of capacitative  $\text{Ca}^{2+}$  entry evoked by thapsigargin (Dammermann et al., 2009). Here, we demonstrate that BZ194 decreases the overall  $\text{Ca}^{2+}$ -response of  $T_{\text{MBP}}$  cells by reducing the percentage of high-responder cells (Fig. 3, pie diagrams and upper panel). Upon preincubation with BZ194, 56% of the T cells showed a low  $\text{Ca}^{2+}$  response after contact to MBP peptide-presenting astrocytes, whereas in vehicle controls only 30% of T cells were low-responders (Fig. 3, pie diagrams). In this series of experiments, without antigen none of the T cells showed an





increase in  $[Ca^{2+}]_i > 300$  nM in the first ten minutes after contact (Fig. 3, pie diagrams and A, upper panel). Although BZ194 shifted the percentage of high-responders from 70% down to 44% (Fig. 3, pie diagrams), it is important to note that there was no further influence of BZ194 on the extent of immobilization and rounding up in BZ194 treated high responder  $T_{MBP}$  cells. Typically,  $T_{MBP}$  cells showed stronger immobilization and rounding up the higher  $[Ca^{2+}]_i$  rose (Fig. 3(B)). The same was observed for  $T_{MBP}$  cells pre-incubated with BZ194 (Fig. 3(C)). Although less of these  $T_{MBP}$  cells showed high  $Ca^{2+}$  responses in the presence

**MESSENGER** 4, 104–111, 2015

## DISCUSSION

Upon IS formation between CD4+ T<sub>MBP</sub> cells and astrocytes we observed (i) rapid T cell Ca<sup>2+</sup> signalling, and (ii) concomitant decrease in cell shape index (rounding) and motility. While the percentage of high-responder T<sub>MBP</sub> cells increased from very few in the absence to about 70% in the presence of antigenic peptide, inhibition of NAADP signalling reduced the percentage down to 44%, thereby partially reverting antigenic stimulation.

Several aspects of the NAADP signalling pathway have been analysed in T cells. In the CD4+ T-lymphoma cell line Jurkat, TCR/CD3 ligation evoked a biphasic increase in NAADP, consisting of a rapid and high rise within the first 10 to 20 sec followed by a much smaller and slower increase over the next minutes (Gasser et al., 2006). Upon microinjection into Jurkat T cells, NAADP stimulated Ca<sup>2+</sup> signalling with a bell-shaped concentration-response curve (Berg et al., 2000). Both initial local as well as global Ca<sup>2+</sup> signals observed upon NAADP administration were sensitive to gene silencing of ryanodine receptors (Langhorst et al., 2004; Dammermann and Guse, 2005). Unexpectedly, evidence for involvement of acidic Ca<sup>2+</sup> stores in NAADP signalling was not obtained in CD4+ Jurkat T cells,<sup>45</sup> though a recent paper reported Ca<sup>2+</sup> release from acidic cytolytic granules of CD8+ T cells (Davis et al., 2012). In rat CD4+ effector T cells NAADP signalling turned out to be a major player in the cellular activation process, likely by delivering trigger Ca<sup>2+</sup> that acts as co-agonist at IP<sub>3</sub>R and RyR (Dammermann et al., 2009). This finding was confirmed in a transfer experimental autoimmune encephalomyelitis (EAE) rat model, often used to mimic aspects of the human disease multiple sclerosis. Cordiglieri et al., showed that treatment of rats with NAADP antagonist BZ194 interfered with movements of effector T cells towards the central nervous system and decreased re-activation of MBP specific CD4+ effector T cells in the brain (Cordiglieri et al., 2010). Importantly, several control experiments in whole animals or rat T cells indicate no obvious side effects of BZ194 suggesting sufficient specificity of the antagonist. Taken, together NAADP signalling plays a pivotal role for activation of CD4+ T cells. Rapid formation of endogenous NAADP and immediate local Ca<sup>2+</sup> signalling upon NAADP microinjection are compatible with the idea that NAADP provides the first increase in [Ca<sup>2+</sup>]<sub>i</sub> that is used then to enhance CICR via IP<sub>3</sub>R and RyR.

IS formation occurred as soon as [Ca<sup>2+</sup>]<sub>i</sub> increased, visible as rapid cellular rounding and the stop of cell motility in our experiments, as demonstrated in Donnadieu et al. (1994). Reorganization of the actin cytoskeleton is a hallmark of IS formation. Signalling proteins involved in this process are the Rho family GTPases Rac1 and Cdc42, and downstream of Cdc42 the Wiskott-Aldrich syndrome protein WASp (Badour et al., 2003). Moreover, downstream of Rac1 WAVE2 and WAVE2 complex proteins,

such as Abi-2 and HEM-1, appear important for actin reorganization since WAVE2 co-localized to IS (Nolz et al., 2006). Furthermore, gene silencing of WAVE2 and HEM-1 reduced the number of cell-cell conjugate formation (Nolz et al., 2006). Moreover, it was demonstrated that under control conditions, Jurkat T cells formed a ring-like lamellipodal interface composed of F-actin on OKT3-coated surface within 1.5 min; upon gene silencing of WAVE2, these ring-like lamellipodal interfaces were not observed resulting in lack of spreading of T cells on the OKT3-coated surface (Nolz et al., 2006). Kaizuka et al., elegantly tracked movements of TCR, ICAM-1 and actin filaments during the process of IS formation (Kaizuka et al., 2007). Importantly, they showed that in IS-forming Jurkat T cells actin speckles moved from peripheral lamella towards the IS in a directed fashion, forming a ring of actin filaments around the central supramolecular activation cluster (cSMAC). Under these conditions, the retrograde flow of actin typical for moving cells was stopped. Instead, T cells used the actin cytoskeleton for cSMAC and peripheral supramolecular activation cluster (pSMAC) formation. Microclusters composed of TCR and ICAM-1 move along underlying cytoskeleton towards the synapse to form cSMAC and pSMAC, though at slower velocity.<sup>37</sup> This shift of actin cytoskeleton action was determined as decreasing cell shape index in our experiments. Since in our experiments NAADP antagonist BZ194 prevented cell rounding and stop of motility, NAADP mediated initiation of Ca<sup>2+</sup> signalling appears a central step in remodelling of the actin cytoskeleton. Although ring-like lamellipodal interfaces were formed rapidly after TCR/CD3 engagement (within 1.5 min; Nolz et al., 2006), we described an even faster biochemical signalling event, the formation of NAADP within 10 to 20 sec upon anti-CD3 mAb stimulation (Gasser et al., 2006). Based on our experimental data, we cannot distinguish whether NAADP evoked, and initially localized Ca<sup>2+</sup> release would be sufficient to shift actin cytoskeletal function from motility to IS formation, or whether NAADP evoked Ca<sup>2+</sup> release is simply necessary to provide a sufficiently high Ca<sup>2+</sup> signal via IP<sub>3</sub>R and/or RyR to induce the shift in actin cytoskeletal function. However, our data indicate a pivotal role of NAADP signalling for IS formation and thus for activation of CD4+ T cells in general.

## Abbreviations

|                                  |  |
|----------------------------------|--|
| APC                              | antigen presenting cell                                |
| [Ca <sup>2+</sup> ] <sub>i</sub> | free cytosolic Ca <sup>2+</sup> concentration          |
| cADPR                            | cyclic ADP-ribose                                      |
| CICR                             | Ca <sup>2+</sup> -induced Ca <sup>2+</sup> -release    |
| c(p)SMAC                         | central (peripheral) supramolecular activation cluster |
| IP <sub>3</sub>                  | D- <i>myo</i> -inositol 1,4,5-trisphosphate            |
| IP <sub>3</sub> R                | D- <i>myo</i> -inositol 1,4,5-trisphosphate receptor   |

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